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Dystroglycan glycosylation and muscular dystrophy

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Abstract Dystroglycan is an integral member of the skeletal muscle dystrophin glycoprotein complex, which links dystrophin to proteins in the extracellular matrix. Recently, a group of human muscular dystrophy disorders have been demonstrated to result from defective glycosylation of the α-dystroglycan subunit. Genetic studies of these diseases have identified six genes that encode proteins required for the synthesis of essential carbohydrate structures on dystroglycan. Here we highlight their known or postulated functions. This glycosylation pathway appears to be highly specific (dystroglycan is the only substrate identified thus far) and to be highly conserved during evolution.

Keywords Dystroglycan · Glycosylation · Muscle · Glycosyltransferase · Muscular dystrophy

Defects in glycosylation of dystroglycan are associated with muscular dystrophy

Dystroglycan plays an integral role within the skeletal muscle dystrophin glycoprotein complex (DGC), which links dystrophin to proteins in the extracellular matrix (Fig. 1). Deficiencies in most of the proteins within this complex result in inherited forms of muscular dystrophy, reviewed in [1]. However, no diseases have yet been associated with mutations in the dystroglycan gene (*DAG1*) itself. Null mutants of *Dag1* in mice show embryonic lethality [2, 3], suggesting that dystroglycan function is essential for embryogenesis. Mature dystroglycan consists of two subunits (α- and β-), produced by post-translational cleavage of a precursor protein [4]. The two subunits bind non-covalently to form a heterodimer within the DGC (Fig. 1). β-dystroglycan is a membrane protein, binding α-dystroglycan via its N terminus and dystrophin through a WW motif within the intracellular domain [5]. Within the DGC, α-dystroglycan functions as a receptor for several extracellular ligands with high affinity, calcium-dependent binding of laminin, perlecan, agrin and neurexin [4, 6–8].

Recently, a group of human muscular dystrophies have been demonstrated to have defective dystroglycan glycosylation and are hence termed dystroglycanopathies [9]. Thus far, six dystroglycanopathy genes have been identified; *POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *FKRP* and *LARGE* [9]. Although a paralogue of *LARGE*, (*LARGE2*) has been identified [10] and is likely to be involved in dystroglycan glycosylation [11–13], no mutations have yet been identified in this gene. Dystroglycanopathies are typically (but not always) severe congenital muscular dystrophy (CMD) and are often associated with neuronal migration defects and ocular abnormalities.

The primary molecular mechanism underlying these muscular dystrophies is hypoglycosylation of α-dystroglycan, which can be seen by loss of immunoreactivity for two monoclonal antibodies (IIH6 and VIA4$_1$) that recognize only functionally glycosylated forms of the protein. This hypoglycosylated form of dystroglycan displays defective ligand binding [14–16], thus destabilizing the link between the cytoskeleton and the extracellular matrix. Conditional depletion of dystroglycan in the central nervous system (CNS) of mice results in neuronal migration abnormalities similar to those seen in these CMDs and in mouse models [17], supporting the hypothesis that loss of functional

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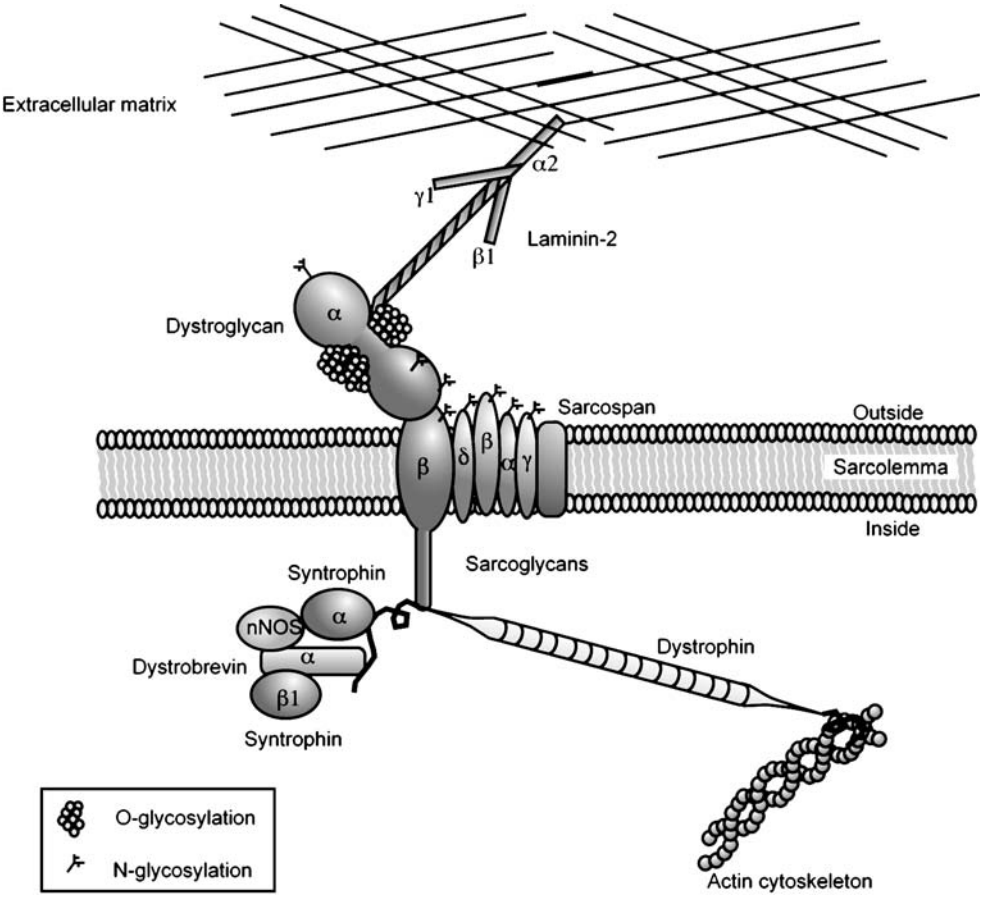

Fig. 1 A representation of the dystrophin-associated glycoprotein complex (*DGC*) in skeletal muscle and some of the associated proteins. The DGC links the extracellular matrix outside the cell with the actin cytoskeleton inside the cell. The key indicates types of glycosylation on dystroglycan

glycosylation of α-dystroglycan is the primary disease mechanism.

Although first identified as part of the skeletal muscle DGC, dystroglycan also has important functions in many non-muscle tissues including basement membrane formation, neuronal migration, and formation of epithelial structures [18]. Some of these non-muscle functions also appear to be disrupted in the dystroglycanopathies, particularly in the CNS. In addition, the protein acts as a pathogen receptor [18] and may play a role in cancer progression [19], with a role for glycosylation also implicated for both these functions.

The dystroglycanopathies

The dystroglycanopathies can be divided into several clinical disorders that range in severity from Walker–Warburg syndrome (WWS), a severe form of congenital muscular dystrophy (CMD) that is also associated with ocular abnormalities and CNS defects [20] to forms of limb girdle muscular dystrophy (LGMD) that have an onset later in life and have no CNS involvement. However, there is little correlation between the particular gene mutated and the clinical picture (Table 1).

WWS is one of the most severe CMDs, with most patients living for one year or less. The disease shows extensive genetic heterogeneity [21], with causal mutations identified in the *O*-mannosyltransferases *POMT1* and *POMT2* [22, 23], and the putative glycosyltransferases fukutin [24], FKRP [25] and LARGE [26]. Based on genetic analysis of families, there are several additional genetic loci for this syndrome [21].

Muscle–eye–brain disease (MEB) is also a severe CMD with additional symptoms including ocular defects, epilepsy and lissencephaly (smooth brain appearance as a result of abnormal neuronal migration). The life expectancy of patients is 10–30 years [27]. The gene most commonly mutated in MEB encodes *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) [28], although like WWS there is genetic heterogeneity with mutations also reported in *FKRP* [25] and *POMT2* [29].

Fukuyama-type congenital muscular dystrophy (FCMD) is an autosomal recessive disorder with a severe muscular dystrophy characterized by muscle weakness, hypotonia and pseudohypertrophy appearing prior to 9 months of age [30]. Patients also have associated brain abnormalities, such as type II lissencephaly, abnormal white matter patterning, cardiomyopathy, and eye abnormalities. FCMD is mostly confined to Japanese populations, where it is the most

Table 1 Genes involved in dystroglycan and dystroglycanopathies

Gene	Associated disorders, key references
POMT1	WWS [22] MEB [33] CMD+MR [78] LGMD2K [79]
POMT2	WWS [23] MEB [29] CMD+MR [33] LGMD2N [80]
POMGnT1	MEB [28] LGMD [42]
Fukutin	WWS [24] FCMD [31] CMD (no MR) [33] LGMD2L [81]
FKRP	WWS [25] MEB [25] CMD+MR [82] CMD (no MR) [33, 81] MDC1C [35] LGMD2I [36]
LARGE	WWS [26] MDC1D [60]

WWS Walker–Warburg syndrome, *MEB* muscle–eye–brain disease, *CMD* congenital muscular dystrophy, *MR* mental retardation, *LGMD* limb girdle muscular dystrophy, *MDC1C* congenital muscular dystrophy type 1C, *MDC1D* congenital muscular dystrophy type 1D

prevalent form of CMD due to a founder effect. Nearly all patients carry at least one *fukutin* allele that has a 3 kb retrotransposon insertion into the 3′UTR. This destabilizes the mRNA and is presumed to act as a hypomorphic mutation, reducing the amount of functional fukutin [31]. Fukutin has a predicted catalytic domain with similarity to phosphoryl-sugar transferases [32].

Very few Japanese FCMD patients have been identified with two point mutations in the coding region, suggesting that complete loss of fukutin may be embryonic lethal. This was thought to explain why FCMD is much less common outside of the Japanese population. In fact, although *fukutin* mutations have been described in a number of non-Japanese patients, some present with a much milder form of dystroglycanopathy that does not involve the CNS [33], while others have the more severe WWS [24, 34].

Congenital muscular dystrophy type 1C (MDC1C) is a severe CMD with a reduced lifespan, muscle weakness and cardiomyopathy, but no associated brain abnormalities or mental retardation. In 2001, MDC1C was shown to be due to mutations in the gene encoding fukutin-related protein (FKRP) [35]. Limb girdle muscular dystrophy type 2I (LGMD2I) a much less severe disease with hypotonia and muscle weakness, sometimes combined with cardiomyopathy, is also due to mutations in FKRP [36]. In both MDC1C and LGMD2I patients, dystroglycan glycosylation is not completely missing in skeletal muscle protein extracts—rather there is a slight reduction in apparent molecular mass [37]. It should be noted that FKRP mutations can result in more severe clinical phenotypes that do include CNS defects [25, 38]; this variability may be related to the residual activity of the mutated protein.

In skeletal muscle, VIA4$_1$ reactive α-dystroglycan is approximately 156kDa, and this immunoreactivity reactivity is lost in muscle extracts from FCMD [39], MEB [40] and WWS [22] patients and in Large^{myd} mice, which have a loss of function mutation in the *Large* gene [14, 15, 41]. In the less severely affected patients with MDC1C and LGMD2I, VIA4$_1$ reactivity is reduced [37]. Using a polyclonal antibody (GT20ADG) against a hypoglycosylated form of α-dystroglycan, Michele *et al*. showed the protein to have a reduced mass in muscle extracts from FCMD and MEB patients and Large^{myd} mice [15]. The signal with this antibody is increased on immunoblots of dystroglycanopathy tissues compared to unaffected controls [15], this may indicate epitope unmasking due to the hypoglycosylation in dystroglycan.

It is clear that most of these clinical disorders are genetically heterogeneous and that the relationship between mutations in a particular gene and the resulting phenotype is very complex. For example mutations in *POMGnT1*, first identified in severely affected MEB patients [28], can also be associated with milder phenotypes such as LGMD [42]. The clinical severity is likely to be related, at least in part, to the extent of dystroglycan hypoglycosylation associated with a particular gene mutation. There are also tissue-specific differences, with the lack of CNS involvement in some patients suggesting that skeletal muscle is more sensitive to perturbation of dystroglycan. In addition, genetic variation between patients for other genes that may have compensatory functions could contribute to this heterogeneity. A recent clinical study indicates that for patients with mutations in *POMT1*, *POMT2* or *POMGnT1*, there is a good correlation between the reduction in skeletal muscle α-dystroglycan glycosylation (as measured by staining with IIH6 antibody) and the disease severity. However, for patients with mutations in fukutin or FKRP (LARGE mutations are extremely rare and were not included) a clear relationship between the level of α-dystroglycan glycosylation and the clinical symptoms was not apparent [43].

Dystroglycan glycosylation

The β-subunit undergoes *N*- and possibly limited *O*-glycosylation [44, 45]. However, α-dystroglycan has extensive and heterogeneous glycosylation. Despite a predicted MW of 72 kDa, on SDS PAGE gels the protein has an apparent mass of 120–190 kDa, due to glycosylation

[6]. Furthermore, the apparent mass varies according to tissue type (156 kDa in mammalian skeletal muscle, 140 kDa in cardiac muscle, and 120 kDa in brain and peripheral nerve), suggesting differential glycosylation [4, 46]. The extent of skeletal muscle α-dystroglycan glycosylation increases during human development [37]. Removal of *N*-glycans with *N*-glycosidase F reduces the apparent mass of the protein by only about 4 kDa [47], indicating that the majority of glycosylation on α-dystroglycan is likely to be *O*-linked. The known glycan structures on dystroglycan have recently been reviewed in detail [44].

Electron microscopy supports a model for α-dystroglycan structure as a rod-shaped central domain bounded on either side by globular regions, giving the protein a dumbbell shape [48]. This central region contains a high number of potential *O*-glycosylation sites (~50) and resembles a mucin domain. Such mucin domains typically have a high density of serine, threonine and proline residues and adopt a stiff extended confirmation due to the steric hindrance of the extensive glycan structures. Thus, one likely function of the α-dystroglycan *O*-glycans is to induce the correct confirmation of the protein. However, they are also proposed to play a more direct role in ligand binding.

It is well established that glycosylation of α-dystroglycan is required for binding to laminin [49]. Chemical deglycosylation of α-dystroglycan purified from rabbit skeletal muscle results in loss of laminin binding [50]. The monoclonal antibody IIH6 (but not $VIA4_1$) blocks laminin binding to α-dystroglycan [46, 51]. N-glycosylation of α-dystroglycan is not required for this ligand binding activity, as affinity is not reduced by extensive *N*-glycanase treatment [50]. Although sialic acids have been implicated as necessary for laminin binding [52], removal of these glycans *in vitro* does not disrupt binding [6, 53] and may even enhance it [54].

Although core-1 type structures (Galβ1-3GalNAcα-*O*-Ser/Thr) are present on α-dystroglycan, there has been much more focus on rare mannosyl *O*-glycans identified by several mass spectroscopy studies, including the structure Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-*O*-Ser/Thr [52, 55, 56]. The reason for this interest is that several of the genes involved in dystroglycanopathy phenotypes are believed to function in the synthesis of these glycans. Co-expression of POMT1 and POMT2 is necessary for *O*-mannosyltransferase activity in mammalian cells, which occurs within the ER [57]. POMGnT1 is a Golgi-resident type-II membrane protein with activity as a protein *O*-mannose β-1,2-*N*-acetylglucosamine transferase [28, 58]. The enzyme adds *N*-acetylglucosamine to *O*-linked mannose via a β1-2 linkage, and is therefore likely also to be involved in the synthesis of *O*-mannosyl glycans on dystroglycan. Thus, genetic data strongly support the hypothesis that the addition of *O*-mannosyl glycans to α-dystroglycan is required for its functional activity as an ECM receptor. However, it is still unclear whether these glycans are directly involved in ligand binding or play some other role.

The function and evolution of LARGE genes

Although POMT1, POMT2 and POMGnT1 have demonstrated glycosyltransferase activity, the biochemical activities of the remaining genes associated with dystroglycanopathy are so far unknown. Although fukutin has a predicted catalytic domain with similarity to phosphorylsugar transferases [32], no enzyme activity has been reported. However, it has been shown to interact with POMGnT1 and therefore may have a chaperone type activity rather than function directly as an enzyme [59]. Similarly, no enzyme activity for FKRP, originally identified by homology to fukutin, has yet been identified. Although mutations in human *LARGE* appear to be a rare cause of dystroglycanopathy, with only two reports to date [26, 60], however, it appears to represent a key enzyme within this dystroglycan glycosylation pathway and therefore will be covered here in more detail.

The role of LARGE in α-dystroglycan glycosylation was first identified when it was shown to be mutated in the myodystrophy (now $Large^{myd}$) mouse, a model of muscular dystrophy [41]. The $Large^{myd}$ phenotype resembles those seen in dystroglycanopathies with a progressive muscular dystrophy, cardiomyopathy, neuronal migration defects, and retinal abnormalities [14, 15], and is associated with hypoglycosylation of α-dystroglycan [15, 41].

The function of LARGE genes

LARGE was originally identified in a study of a region of chromosome 22 with a high frequency of tumour-specific interstitial deletions, particularly in meningioma [61]. Despite only 2 kb of coding sequence, the 16 exons of the human gene cover a region of over 660 kb of genomic DNA (hence the name). By northern blot, *LARGE* mRNA is widely expressed with highest levels in heart, brain and skeletal muscle [61]. The *LARGE2* paralogue is not expressed in muscle and brain, but transcripts are found in other tissues, particularly testis and kidney [13]. LARGE proteins have two predicted catalytic domains [41]. The first catalytic domain belongs to the GT-8 family, which contains α-glucose/galactose transferases [62]. The second catalytic domain belongs to the GT-49 family and shows highest similarity (33% amino acid identity) to iGnT, a β-1,3-*N*-acetylglucosaminyltransferase involved in the syn-

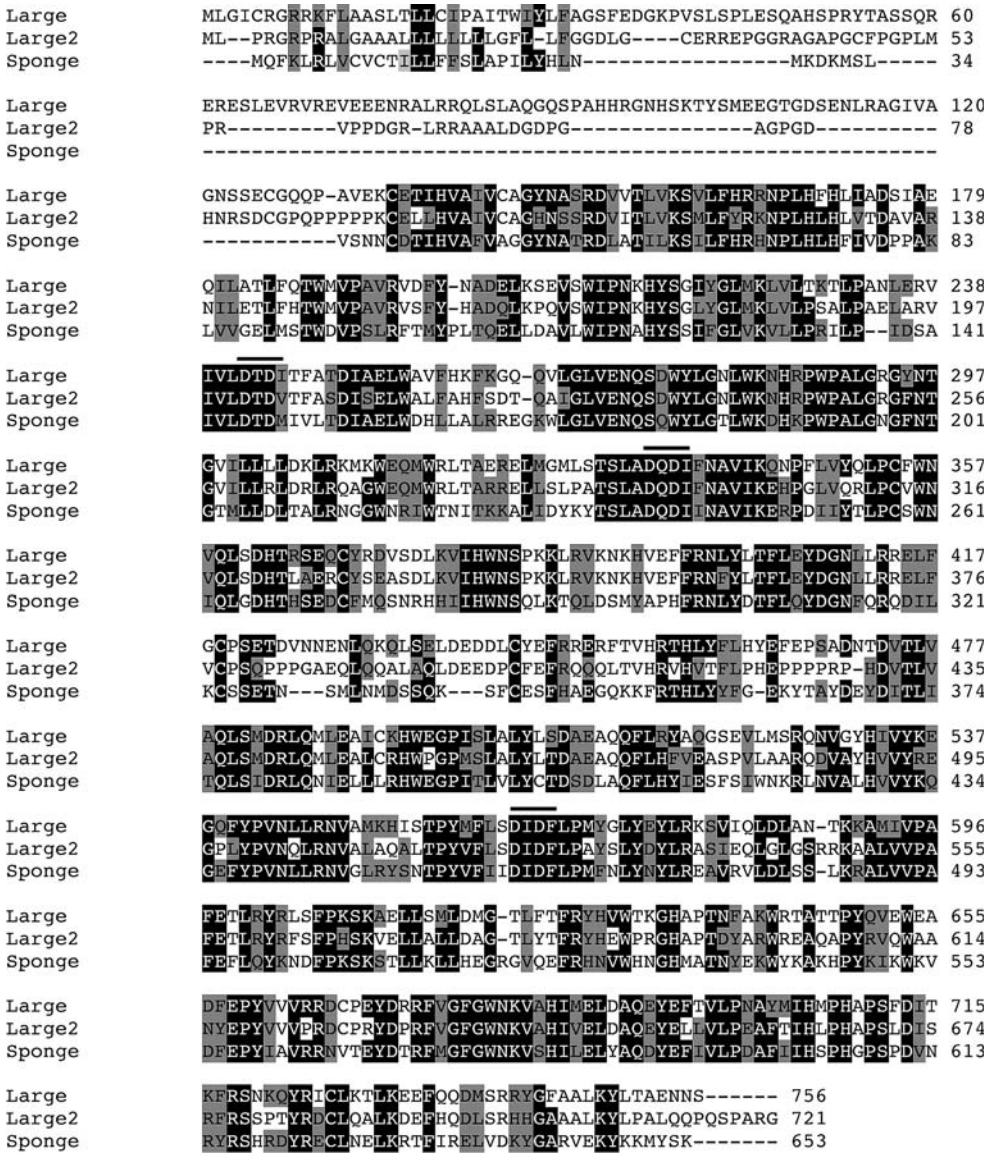

Fig. 2 Amino acid alignment of mouse and sponge Large proteins. Manually edited ClustalW amino acid alignment of the amino acid sequences of mouse Large and Large2 and the predicted *Reniera* protein. Residues conserved between the *Reniera* protein and at least one of the mouse proteins are shaded: *white letters on black* = identical, *white letters on dark grey* = conserved. *Horizontal bars* above the alignment represent the locations of the three DXD domains

thesis of poly-*N*-acetyllactosamine on the erythrocyte i antigen [63]. Both catalytic domains contain DXD motifs flanked by hydrophobic residues (in fact the GT-8 domain has two), which are often characteristic of glycosyltransferases [64].

Although it has not yet been demonstrated that LARGE proteins have glycosyltransferase activity themselves, overexpression of either paralogue in cultured cells is able to induce hyperglycosylation of α-dystroglycan and concomitant laminin-binding [11–13, 65, 66]. This *in vivo* activity is characterised by induction of a higher mass form of the protein that is immunoreactive for both IIH6 and VIA4$_1$. Both LARGE and LARGE2 localise to the Golgi apparatus [13]. The short cytoplasmic tails of these proteins contains a basic motif similar to the [RK]X[RK] export motif previously reported to be required for Sar1 binding and export from the ER [67]. Through deletion analysis and site-directed mutagenesis of this domain, we have shown that these motifs are required for correct Golgi localisation (unpublished data). Thus, LARGE proteins show a sub-cellular distribution and biological activity *in vivo* consistent with the hypothesis that they act as glycosyltransferases.

A striking and possibly highly significant finding was that LARGE can induce hyperglycosylation of α-dystroglycan in cells that are defective for *fukutin* or *POMGnT1* [65]. This suggest that the protein acts (or can act) in a different glycosylation pathway to the other dystroglycanopathy genes and identified a potential therapeutic pathway that might be generally applicable to the dystroglycanopathies [9]. Using mass spectroscopy analysis, no absent or abnormal glycans were detected in the Large^{myd} mutant [68], indicating that LARGE may be responsible for addition of a rare carbohydrate structure. Consistent with

this, LARGE is also able to hyperglycosylate α-dystroglycan in CHO cell lines that are deficient for enzymes within key N- or *O*-glycosylation pathways [66].

The evolution of LARGE genes

Phylogenetic and genomic analysis indicated that *LARGE* and *LARGE2* are paralogous genes that originate from a gene duplication event in the vertebrate lineage [13]. The mammalian LARGE orthologues all have a similar exon organization with gene sizes of 300–500 kb. The *LARGE2* paralogues, in contrast, span only 5–7 kb despite a similar number and size of exons to the LARGE genes [13]. Althought his difference in gene size between paralogues is particularly striking for mammals, it is also seen in chicken, *Xenopus tropicalis*, *Fugu rubripes*, and *Tetraodon nigroviridis*. Examination of the evolutionary origins of gene paralogues has shown that the larger of the paralogues are generally the ancestral gene and tend to be located in gene poor regions that contain more LINE sequences and have a lower GC content [69, 70]. *LARGE* orthologues lie within gene poor regions containing LINES, while the *LARGE2* orthologues are present in regions with a higher density of compact genes. The structure of the *LARGE* gene may result in susceptibility to deletion mutations; two spontaneous genomic deletion mutations have identified in mouse *Large* [41, 71] and a human WWS patient is homozygous for an intragenic deletion in *LARGE* [26].

Most invertebrates contain a single *LARGE* gene. One exception is *Drosophila*, where no *Large* homologue could be identified [13], although orthologues of *POMT1, POMT2* and *FKRP* genes are all present in *Drosophila* species. Although *POMT1* and *POMT2* are required for proper muscle integrity and function in *Drosophila* [72], the follicle cell epithelium contains a splice form of dystroglycan that lacks the mucin-like domain but is still able to bind perlecan and affect laminin localization [73]. Therefore, it is possible that the mucin-like domain (and hence its glycosylation) is not essential for all dystroglycan ligand binding in *Drosophila*. Alternatively, if Large-type glycosyltransferase activity is present in *D. melanogaster*, separate GT-8 and GT-49 family enzymes may confer it.

With this possible exception of *Drosophila*, dystroglycan glycosylation appears highly conserved during evolution. For examples, all the mammalian genes in this pathway are conserved in zebrafish, as are the VIA4$_1$ and IIH6 epitopes [74, 75], indicating that the zebrafish is another potential model system for these disorders. By examining DNA sequence information from whole genome projects we have identified *LARGE* homologues throughout the animal kingdom, including Cnidarians and sponges. For this analysis, we defined homologues as genes encoding proteins containing both a GT-8 and a GT49 glycosyltransferase domain. Although the *Dictyostelium discoideum* genome has been reported to contain LARGE homologues [76], the predicted proteins contain only the GT-8 domain and thus are unlikely to be true orthologues. We were interested to find that the genome of the demosponge *Reniera* appears to contain a single (intronless) *LARGE* gene. An alignment of the predicted amino acid sequence of the *Reniera* protein compared to the mouse Large and Large2 proteins is shown in Fig. 2. Both predicted catalytic domains show a very high level of amino acid conservation, with all three DXD motifs present within the sponge protein. Thus, LARGE is a very ancient protein as demosponges are part of the most ancient and basal metazoan lineage [77]. It is not yet clear if LARGE has a role in dystroglycan glycosylation in these evolutionarily distant organisms or whether this represents a function that evolved more recently.

Summary and perspectives

Thus, at least seven genes are involved in functional glycosylation of dystroglycan. Although mutations in *LARGE2* have not been identified in muscular dystrophy, the gene is expressed at very low levels in skeletal muscle and the CNS [13], and therefore such mutations are likely to result in a very different phenotype. Dystroglycan appears to be the major target for these rare modifications, such as the *O*-mannosyl and the unknown LARGE-induced glycans, although it would seem likely that there are others. Identification of single LARGE homologues in all animal groups suggests that the glycosylation pathway is very ancient. Identification of the glycan structures induced by LARGE activity, their relationship to laminin binding and the IIH6/VIA4$_1$ epitopes and the determination of the enzyme activity of LARGE remain significant hurdles to progress in understanding dystroglycanopathies.

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